



PATENT
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Applicant: Kinneret SAVITZKY et al. Ccnf.: 6574
Appl. No.: 10/031,607 Group: 1647
Filed: June 12, 2002 Examiner: HAMUD, F.M.
For: SPLICE VARIANTS OF CD40-RECEPTOR

DECLARATION UNDER 37 C.F.R. § 1.132

Commissioner for Patents
P.O. Box 1450
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Sir:

I, Amir TOPORIK, residing at 124 Simtat Kordova, Mishma Hashiva, ISRAEL, declare as follows:

1. I am a citizen of ISRAEL.
2. I am an inventor of subject matter in the same protein family as the disclosure in U.S. Application Serial Number 10/031,607
3. I am currently employed in research activities at Complugen Ltd. I am engaged in research on alternative splice variants of CD40 receptors. I am an author or co-author of papers in this field. A copy of my curriculum vitae is attached.

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4. I have read and understand the subject matter of U.S. Application Serial Number 10/031,607 and I am familiar with the prosecution of the application.

5. I have carried out, or supervised, the experiments relating to the invention described below.

6. The following experimental data shows that the novel sequences of the invention disclosed in U.S. Application Serial Number 10/031,607 have biochemical utility. In particular, the data demonstrates that while a soluble truncation variant of the wild type CD40 receptor, lacking the unique sequence beginning at amino acid 136 of the sequence depicted in SEQ ID NO: 7, resulted in a decreased level of secretion of the cytokine RANTES, the administration of the CD40 splice variant of the present invention resulted in an increase in RANTES secretion. This finding shows that the splice variants of human CD40 disclosed in the instant specification retain their ability to bind ligand, and thusly have a well-established utility.

7.

Experimental data shows that the novel sequence of the invention regarding the CD40 splice variant according to SEQ ID NO:7 has an unexpected biochemical effect. In particular, the data demonstrate that while a soluble truncation variant of the known CD40 receptor, lacking the unique sequence beginning at amino acid 136 of the sequence depicted in SEQ ID NO:7, resulted in a decreased level of secretion of the cytokine RANTES, the administration of the CD40 splice variant of the present invention resulted in an increase of RANTES secretion.

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EXPERIMENTAL PROCEDURES

Materials and Methods

The CD40 splice variant of the present invention was prepared according to SEQ ID NO:7. The CD40 known extracellular domain sequence (for the comparison soluble CD40 that is known in the art) was amplified by PCR such that the transmembrane domain of the known CD40 protein was excluded, without any additional unique tail.

Fused constructs were created, in which the Fc chain of Immunoglobulin IgG was fused downstream from the CD40 protein (either downstream of Skip 5 (CD4 variant according to the present invention), or downstream of the WT CD4 (known CD40 soluble variant, also described below as sCD40)). Fused proteins of receptor molecules and the Fc of immunoglobulins have been shown to have greater influence on transmembrane signaling-related pathways than unfused receptor molecules, presumably by creating receptor dimers which are more stable than monomers (KM Mohler, et al., J. Immunol, 151, (3) 1548-1561, 1993). Addition of an Fc chain to various CD40 proteins has been shown to increase the lifetime ($T_{1/2}$) of the construct, and to simplify the protein extraction procedure.

EXAMPLE 1: FACS analysis of sCD40 binding to mouse fibroblasts expressing human CD154

10^6 mouse fibroblasts (stably transfected with full length human CD154) were incubated with either sCD40 WT (panel B, below) or skip5 (panel A, below). All were Fc tagged, and present at a concentration of 1- 50 $\mu\text{g/ml}$ at 4°C for 60 min in total volume of 100 μl (0.5×10^6 cells). As a control, we used mouse fibroblasts which do not express CD154 (panel C, below). sCD40 binding was detected using PE-conjugated anti CD40 non-blocking antibody (clone EA-5). Analysis was performed using BD FACsCalibur.

The controls included the following:

1. mouse fibroblast not expressing hCD154.

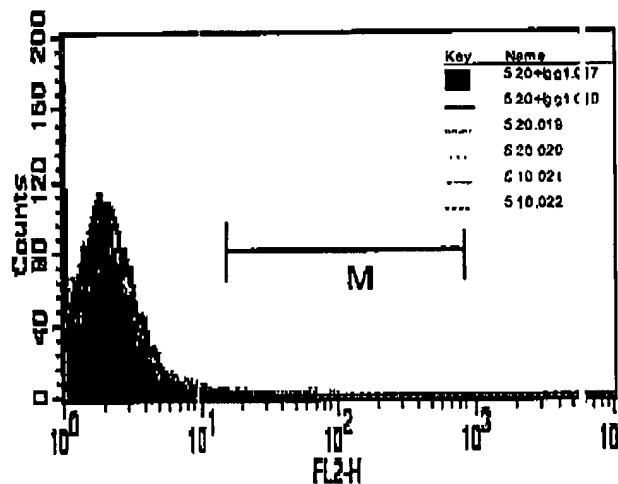
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2. Non-relevant Fc or non-Fc tagged proteins or purificat on mock
3. Isotype control with sCD40
4. Secondary Ab only and isotype control only.

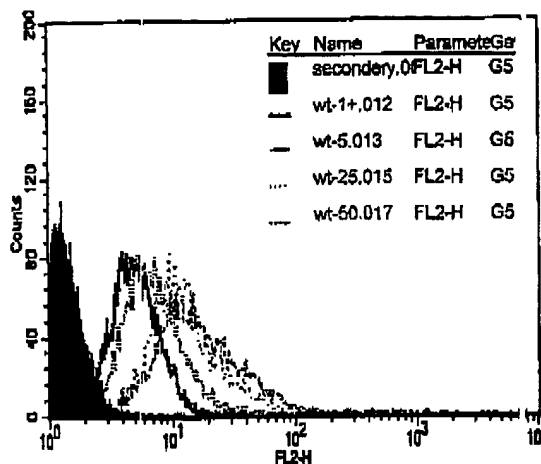
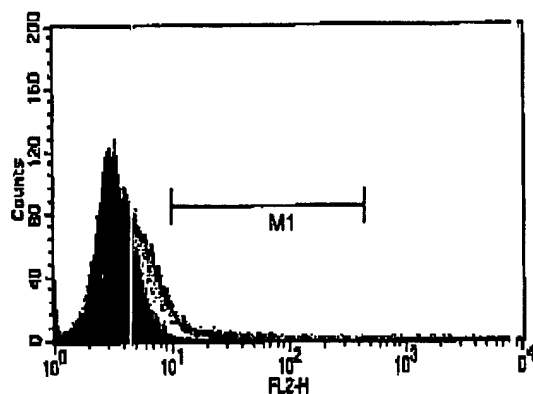
Results of FACS analysis: sCD40-Fc binding to mouse L-fibroblasts transfected with human CD154

The following refers to panels A, B and C below. Y axis: cell counts; X axis: log scale of fluorescence intensity. M indicates a marker placed above the peak of positively stained cells on the histogram plot which provide the statistic of stained population.

A



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B**C**

The FACS experiment showed significant binding of WT-sCD40-Fc to membrane CD154 while the Skip5-sCD40-Fc exhibited different properties. This could be explained by different binding/functional properties of this variant (see RANTES assay).

EXAMPLE 2: Effect of the CD40 variant on RANTES secretion

Skip 5 protein (CD40 splice variant of the present invention) was administered to a mixture of human peritoneal cells (HPMC cells), which express the CD40 receptor on their membrane, and mouse fibroblasts transfected to express the CD154 ligand. The ability of the soluble skip 5 protein

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to compete with the CD40 membrane-bound receptor for binding to the CD154 ligand presented on the mouse fibroblasts was thus tested.

This ability was measured by determining the resultant level of the cytokine RANTES, which is a cytokine indicative of T cell activation, as compared to administration of a positive control (interferon, which raises the level of RANTES via a CD40-related pathway).

The resultant concentration of RANTES under each experimental condition was measured with a standard ELISA test.

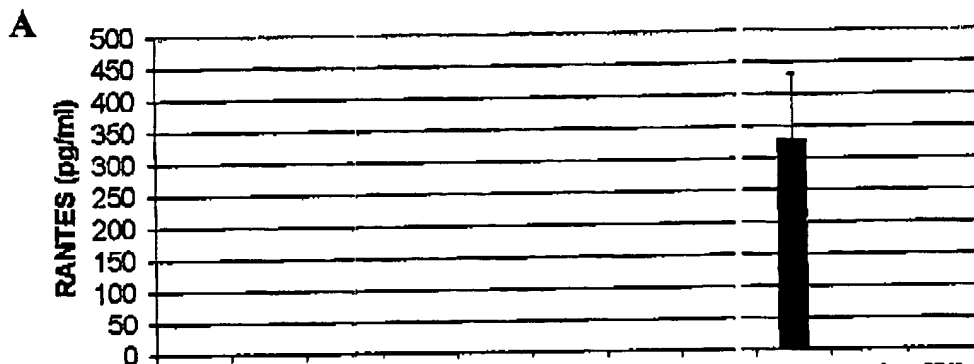
Results:

Graphs A and B below show results for experimental conditions in which there were only HPMC cells, or the mouse fibroblasts used in conjunction with the HPMC cells were not transfected and thus did not express CD154. There was therefore no ligand present in the system, and RANTES secretion could not be activated via the CD40-CD154 pathway. The level of RANTES was thus relatively low except for reactions where Skip5-sCD40-Fc (CD40 splice variant of the present invention) was present and exhibited an agonistic effect. This effect was found to be interferon dependent but CD154 independent.

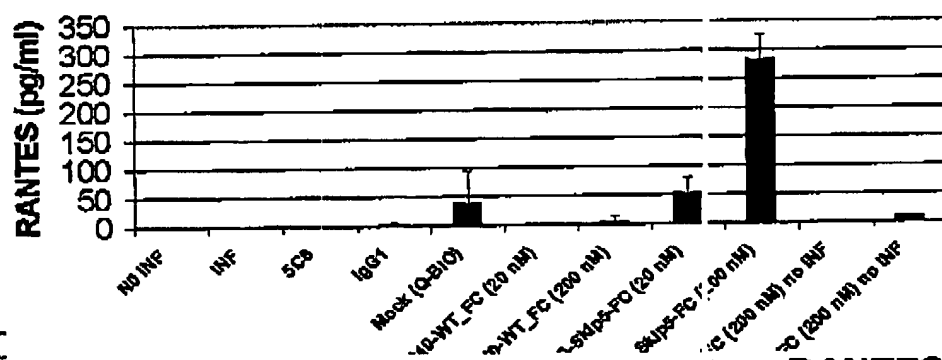
Referring to Graph C, the mouse fibroblasts used in conjunction with the HPMC cells were transfected to express CD154. Both ligand and receptor were present, therefore administration of the positive control interferon (INF) activated the CD40 pathway, and raised the RANTES level to 2000 pg/ml. Administration of the antibody 5C8 (a blocking antibody) and WT-sCD40-Fc (soluble CD40 that is known in the art) lowered this level to approximately 1000 pg/ml (50 % inhibition), while Skip5-sCD40-Fc showed no inhibition in the complete system. The controls (hIgG1 and mock) did not influence any of the above results.

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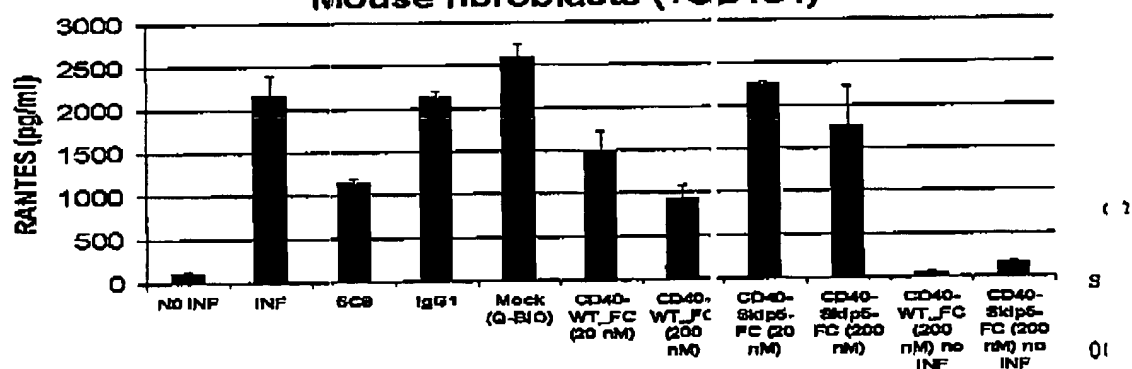
Influence of proteins treatments on RANTES secretion by HPMCs only



B Influence of proteins treatment on RANTES secretion in a system composed of HPMCs and Mouse fibroblasts (-CD154)



C Influence of proteins treatment on RANTES secretion in a system composed of HPMCs and Mouse fibroblasts (+CD154)

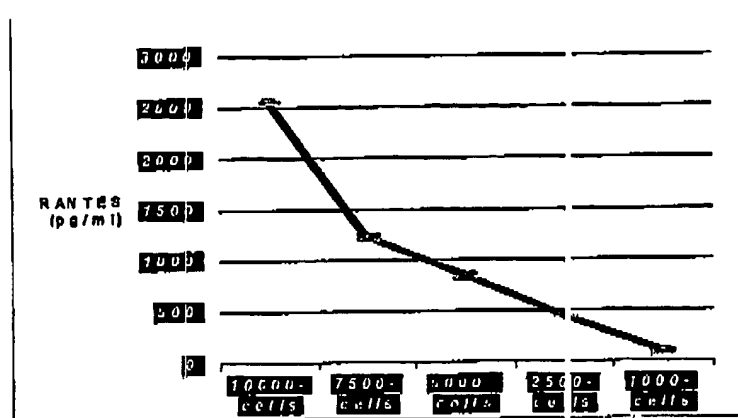


%), while Skips-CD40-Fc exhibited a stimulatory trend.

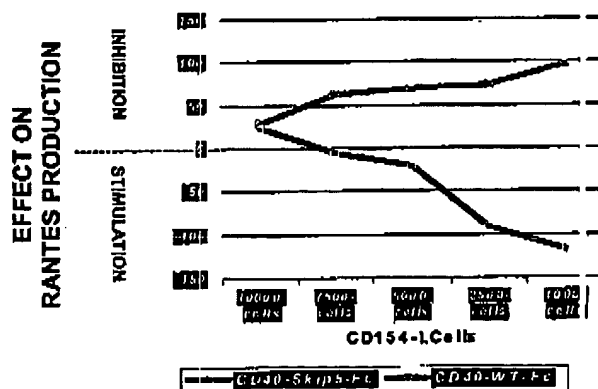
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D

Titration of stimulated RANTES production by varying CD154+ mouse fibroblasts number

**E**

Contrasting effects of stimulated RANTES production by sCD40 w/t and skip-5 Fc proteins



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I hereby declare that all statements made herein of my own knowledge are believed to be true, and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Dated this 22 day of November, 2004

Amir TOPCHIK
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Chemokines activate natural killer cells through heterotrimeric G-proteins: implications for the treatment of AIDS and cancer

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ABSTRACT Natural killer (NK) cells are anti-tumor and anti-viral effector cells. These cells show increased cytolytic activity upon stimulation with interleukin 2 or chemokines. In addition, members of the C, CC, CXC, or CX₃C chemokines induce the *in vitro* chemotaxis of NK cells and contribute to their *in vivo* tissue accumulation. Chemokines induce various intracellular signaling pathways in NK cells by activating members of the heterotrimeric G-proteins. Understanding these pathways should provide an insight into NK cell activation, *in vivo* distribution, and tissue localization. Based on evidence showing the high lytic activity of these effector cells against transformed or virally infected cells, it is suggested that NK cells can be used to maximize the immunotherapeutic protocols for AIDS and cancer patients.—Maghazachi, A. A., Al-Aoukaty, A. Chemokines activate natural killer cells through heterotrimeric G-proteins: implications for the treatment of AIDS and cancer. *FASEB J.* 12, 913–924 (1998)

Key Words: signaling pathway · HIV-1

BACKGROUND

Natural cytotoxicity is the process of spontaneous destruction of cells infected with noxious agents such as bacteria, viruses, protozoans, and other parasites. In the early 1970s, a new type of cell was discovered and designated natural killer (NK)² cell. NK cells mediate the natural cytotoxicity process and are the major components of the immune surveillance mechanism, in which the immune cells recognize and kill both infected and transformed cells (reviewed in ref 1). The potential effect of NK cells in destroying tumor cells without presensitization was the hallmark of the field of natural immunity. To perform this function, these cells must adhere, migrate, and produce anti-microbial and anti-tumor activities. These cellular responses occur under the influence of specific ligands and depend on a series of consecutive events. Investigators in this field examined the migratory behavior of NK cells with the intention of maximizing their localization at the sites of infection

or at tumor growth sites. The majority of ¹¹¹indium-labeled rat NK cells were localized in the liver, lungs, and spleen after systemic administration. These cells were not found in the peripheral lymph nodes (PLN) or splenic white pulp, which are predominant areas where T and B lymphocytes lodge (2). These findings suggest that adoptively transferred NK cells do not localize into the primary lymphoid tissues. However, considerable evidence suggests that NK cell activity can be modulated in both lymphoid and nonlymphoid organs such as the liver after administration of biological response modifiers (3). Administration of interferon gamma (IFN- γ) into the peritoneal cavity of mice causes an infiltration of NK cells into this area (4). These results demonstrate that although NK cells do not recirculate between blood and lymphoid tissues, they can be induced to infiltrate into tissue sites. However, the nature of the chemoattractants and the mechanisms of recruitment of NK cells toward the inflammatory sites need further examination.

A characteristic of NK cells is their expression of interleukin 2 receptors (IL-2R). At least three receptors for IL-2 are present on lymphoid and some non-lymphoid cells. These receptors are designated as IL-2R α , IL-2R β , and IL-2R γ (5). The majority of NK cells express IL-2R β (6). Because IL-2R β is capable of transducing the intracellular signals of IL-2, it is feasible to activate NK cells in the presence of IL-2 only,

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² Abbreviations: NK, natural killer; PLN, peripheral lymph nodes; IFN, interferon; IL, interleukin; LAK, lymphokine-activated killer; A-LAK, adherent LAK; ERK, extracellular-regulated kinase; TNF, tumor necrosis factor; MIP, macrophage inflammatory protein; MCP, macrophage chemotactic protein; RANTES, regulated upon activation, normal T cell expressed and secreted; IP, interferon-inducible protein; SDF, stromal-derived factor; MDC, macrophage-derived chemokine; CMV, cytomegalovirus; G-protein, guanine nucleotide binding protein; CT, cholera toxin; PT, pertussis toxin; cAMP, cyclic adenosine monophosphate; SLO, streptolysin O; GPCR, G-protein-coupled receptors; RTK, receptor tyrosine kinases; MAP, mitogen-activated protein kinase; PI-3K, phosphoinositide-3 kinase; SH, Src homology; CHAK, chemokine-activated killer; IANK, IL-2 activated NK.

and without antigenic stimulation. IL-2-activated cells are designated as lymphokine-activated killer, or LAK cells (reviewed in ref 7). These cells can kill a variety of tumor-cultured cells and fresh explanted tumors. Consequently, LAK cells have been used to treat cancer patients with melanoma, renal cell carcinoma, and colorectal cancer. A substantial response and convincing regression of sizeable metastatic lesions have occurred in some patients. Other studies strongly implicate NK cells in combating virally infected cells through either IFN- γ secretion or through direct contact with virally infected cells (8, 9).

Despite their potential anti-tumor activity, two major problems became apparent: 1) administration of LAK cells into cancer patients in conjunction with IL-2 resulted in substantial toxicities due to injuring the vascular endothelium as a consequence of the administration of high doses of IL-2, and 2) LAK cells do not migrate toward the sites of tumor growth (reviewed in ref 10). Therefore, the objectives are to devise effective protocols for such therapy and to focus on strategies to dissociate the therapeutic and toxic effects. This could be achieved by generating a purified population of LAK effector cells that could be administered in conjunction with small concentrations of IL-2, and by redirecting the effector cells toward the sites of tumor growth so that they can come into direct contact with metastatic tumor cells and to induce their destruction.

Vujanovic et al. (11) purified a population of LAK effector cells through adherence to plastic flasks after incubation with IL-2. Most if not all of these cells showed the morphology and phenotype of NK cells. These cells are designated as adherent LAK (A-LAK) cells or as IL-2-activated NK (IANK) cells (11, 12). IANK cells demonstrate high anti-tumor activity, show strong ability in eliminating both micro- and macrometastases, and significantly prolong the survival of mice treated with them (13, 14). Redirecting NK cells toward the sites of tumor growth is more difficult to achieve because of their inability to extravasate into various organs (15–17). This prompted us and others to examine the capacity of cytokines to induce the motility of NK cells and to investigate the mechanisms by which cytokines activate the migratory signals in these cells. For this purpose, we used the in vitro microchemotaxis assay of Boyden. We reported that tumor necrosis factor α (TNF- α) is a chemokinetic factor for rat IANK cells (18). However, TNF- α and other cytokines such as IFN- γ and IL-1 induce the secretion of various chemotactic factors, including IL-8 (19), which is secreted by several cell types (20). This cytokine induces the in vitro chemotaxis of neutrophils and T cells (21). Therefore, we examined whether IL-8 induces the locomotion of IANK cells and reported that IL-8 induces the random

TABLE 1. Members of the chemokine family and their effects on NK cell chemotaxis^a

	Chemotaxis (Reference)
CXC	
IL-8	– (12), – (29), – (30)
NAP-2	NE
GRO- α	NE
GRO- β	NE
GRO- γ	NE
IP-10	+ (29), – (30), + (31)
SDF-1 α	+ (34)
SDF-1 β	NE
PF4	NE
MIG	NE
ENA	NE
BCA-1	NE
CC	
MIP-1 α	+ (27), + (29), + (30)
MIP-1 β	– (27), + (29), + (30)
MCP-1	+ (27), + (28), + (29), + (30)
MCP-2, -3	+ (28), + (29), + (30)
MCP-4, -5	NE
Eotaxin	NE
RANTES	+ (27), + (29), + (30)
TCA (I-309)	– (30)
MIP-3 α (exodus, LARC)	+ (Unpublished results)
MIP-3 β (ELC)	+ (Unpublished results)
MDC	+ (35)
6Ckine (exodus 2)	NE
TECK	NE
TARC	NE
PARC (DC-ck1)	NE
Leutactin	NE
C	
Lymphotactin	+ (31), + (32), + (33)
CX ₃ C	
Fractalkine	+ (36), + (Unpublished results)

^a This table represents a collection of data published on chemokine-induced NK cells motility. No distinction has been made between freshly isolated, IL-2-activated, or CC-chemokine activated NK cells. Generally, IL-2-activated NK cells respond better than freshly isolated cells. In most cases, chemokines induce the chemotaxis and not the chemokinesis of NK cells, except for IL-8, which induces the chemokinesis of IANK cells (12), and MCP-1 or RANTES which induce the chemokinesis of the NK 3.3 cell line, but not IANK cells (27). (+) Indicates a response; (–) indicates no response. Abbreviations: CXC chemokines: BCA-1, B cell-attracting chemokine 1; ENA, epithelial-derived neutrophil chemoattractant; GCP-2, granulocyte chemotactic protein 2; GRO, growth related oncogene; IL-8, interleukin 8; IP-10, interferon-inducible protein 10; MIG, monokine induced by interferon; NAP-2, neutrophil activating protein 2; PF4, platelet activating protein; SDF-1, stromal-derived factor 1. CC chemokines: ELC, EBV ligand chemokine; LARC, liver and activation-regulated chemokine; MCP, macrophage chemotactic protein; MDC, macrophage-derived chemokine; MIP, macrophage inflammatory protein; PARC, pulmonary and activation-regulated chemokine; RANTES, regulated upon activation, normal T cell expressed and secreted; TARC, thymus and activation-regulated chemokine; TCA, T cell activation gene; TECK, thymus-expressed chemokine. NE, not examined.

motility of human IANK cells (12). Because IL-8 did not induce the chemotaxis of NK cells, a search was undertaken to find other mediators that may perform this function. Due to the potent chemotactic activity of other chemokines for T and B lympho-

cytes and their potent roles in eliciting and maintaining the inflammatory responses, we sought to determine their chemotactic activity for NK cells.

Chemotactic cytokines (chemokines) are proinflammatory mediators necessary for the recruitment of various cell types to the inflammatory sites (reviewed in refs 22–25). They mediate the allergic disorders, autoimmune diseases, and ischemia associated with the infiltration of leukocytes (26). Depending on the presence and arrangement of the first cysteine residues in the NH₂-terminal region, chemokines are divided into four subfamilies: CXC (α), CC (β), C (γ), and CX₃C (δ) (Table 1). Examination of the role of chemokines revealed that the CC chemokines macrophage inflammatory protein (MIP-1 α), macrophage chemotactic protein (MCP-1), and regulated upon activation, normal T cell expressed and secreted (RANTES), but not MIP-1 β , induce the chemotaxis of NK cells (27). Allavena et al. (28) reported that MCP-1, -2, and -3 induce the chemotaxis of these cells. Later work by others (29, 30) supports these findings. Recently, the C chemokine lymphotactin (31–33), the CXC chemokine IP-10 (interferon-inducible protein 10) (29, 31), the CXC chemokine SDF-1 α (stromal-derived factor 1 α) (34), the CC chemokine MDC (macrophage-derived chemokine) (35), the CC chemokine MIP-3 α and MIP-3 β (unpublished observations), and the CX₃C chemokine fractalkine (36; unpublished observations) have been shown to induce chemotaxis of NK cells. Table 1 summarizes the current knowledge regarding NK cell chemoattraction by chemokines. Recently, Salazar-Mather et al. (37) showed that MIP-1 α recruits NK cells toward the livers of cytomegalovirus (CMV)-infected mice, resulting in increased inflammation and decreased susceptibility to infection with this virus. Furthermore, Hedrick et al. (33) reported that the administration of lymphotactin into the peritoneal cavity of mice resulted in the accumulation of many NK cells. It was observed that NK cells accumulate in the uterus of pregnant mice (38). Whether there are unique chemokines responsible for the accumulation of NK cells at these sites has not been explored, but is an important issue that needs to be investigated. Taken together, the evidence is overwhelming regarding the chemoattraction effects of chemokines for NK cells.

CHEMOKINES ACTIVATE G-PROTEINS IN NK CELLS

IL-8 receptors were the first CXC chemokine receptors cloned (39, 40). These receptors contain seven hydrophobic segments that span the plasma membrane (seven transmembrane-spanning domains), a characteristic of the superfamily of guanine nucle-

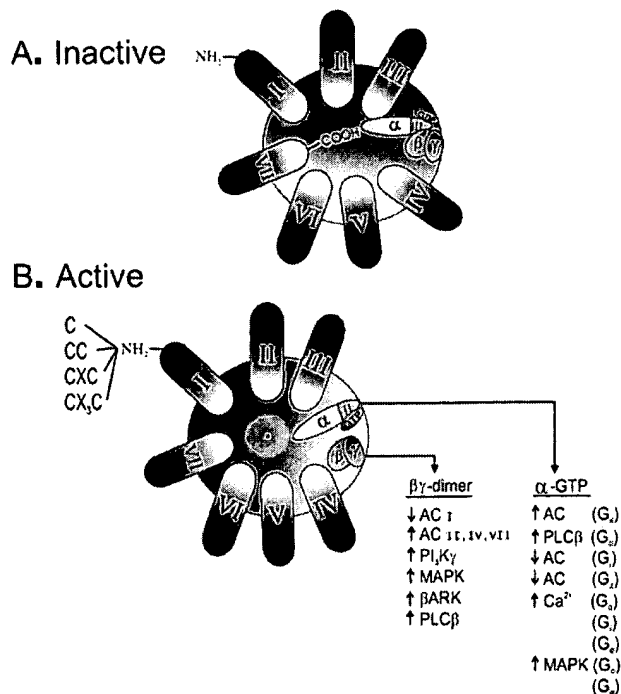


Figure 1. Structure of G-protein-coupled chemokine receptors. Cross section of NK cell surface showing chemokine receptors composed of amino acid segments that traverse the membrane seven times (I–VII loops). In its inactive form, the third cytoplasmic segment binds the carboxy terminal of the α subunit of G-protein. The switch II region of the amino terminal of the α subunit binds the $\beta\gamma$ dimer as well as GDP. Upon chemokine binding to their putative receptors (usually through amino terminal/amino terminal interaction; some chemokines also bind the extracellular domains of the receptor), GTP binds the switch II region and displaces the $\beta\gamma$ dimer from the α subunits. Both these subunits activate various second messengers (\uparrow indicates activation or association, \downarrow indicates inhibition). This model is based on papers published by various investigators (50, 51, 53–55).

otide binding protein (G-protein) receptors. Similarly, receptors for CC chemokines are coupled to G-proteins (41, 42). G-proteins act as mediators between events at the outer surface of target cells and various intracellular effectors that ultimately pass the orders to the final executors. The heterotrimeric G-proteins are composed of three subunits: α , β , and γ . About 20 α , 6 β , and 12 γ subunits have been reported (43). The α subunits belong to four subfamilies: 1) α_s (α_s and α_{olf}); 2) α_q (α_q , α_{11} , α_{14} , α_{15} , and α_{16}); 3) α_i (α_{i1} , α_{i2} , α_{i3} , α_{o1} , α_{o2} , α_z , α_{i1} , α_{gus} , α_{con} , and α_{rod}); and 4) α_{12} (α_{12} and α_{13}). Members of G_q, G₁₂, and G_z differ from other G-proteins by being insensitive to bacterial toxins. Cholera toxin (CT) ADP-ribosylates the arginine residue present in the carboxy terminal of the α subunit of G_s, whereas pertussis toxin (PT) ADP-ribosylates the cysteine residue in the carboxy terminal of the α subunit of G_i or G_o (reviewed in ref 44). Each G-protein has a unique biological function; for example, G_s activates adenylyl cyclase, resulting in

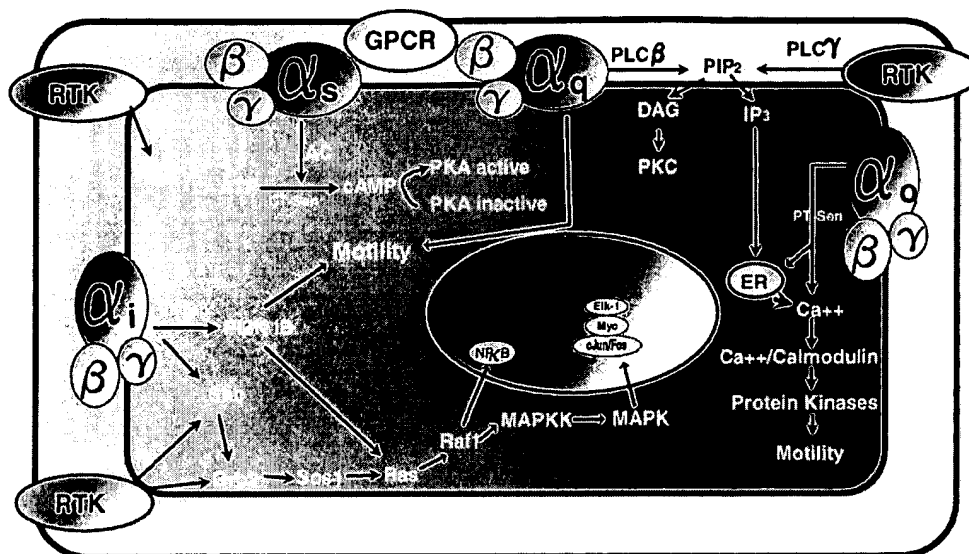


Figure 2. Intracellular signaling pathways in NK cells. Multiple signaling pathways are activated upon ligation of receptors present on these cells. The signals are transmitted through either RTK or GPCR. The $[Ca^{2+}]_i$ pathway can be activated by either RTK/PLC γ or G_q /PLC β . This will result in the hydrolysis of PIP $_2$ into two second messengers: IP $_3$, which induces the mobilization of $[Ca^{2+}]_i$ from the ER, and DAG, which activates PKC. The p21^{ras}/MAP kinase pathway results from the activation of either RTK or GPCR. MAP kinases phosphorylate various nuclear binding proteins such as c-Jun/Fos, c-Myc, and ELK-1, among others. PI-3K is present in one of two forms: PI-3KIA (p110 and p85) binds the receptors upon tyrosine phosphorylation, whereas PI-3KIB (p110 and p101) binds the GPCR upon activation. Abbreviations for Figs. 1 and 2: AC, adenylyl cyclase, cAMP, cyclic adenosine monophosphate, β ARK, beta adrenergic receptor kinases, CT, cholera toxin, DAG, diacyl glyceride, ER, endoplasmic reticulum, GPCR, G-protein-coupled receptors, Grb-2, growth factor receptor bound protein 2, IP $_3$, inositol 1,4,5-trisphosphate, MAPK, mitogen-activated protein kinase, PI-3K, Phosphoinositide-3 kinase, PIP $_2$, phosphatidylinositol bisphosphate, PKA, protein kinase A, PKC, protein kinase C, PLC, phospholipase C, PT, pertussis toxin, RTK, receptor tyrosine kinases, Shc, Src homology and collagen, SOS, son of sevenless.

the accumulation of cyclic adenosine monophosphate (cAMP), whereas G_i inhibits the accumulation of cAMP. The bacterial toxin-insensitive G_q activates phospholipase C β , resulting in the hydrolysis of phosphatidylinositol bisphosphate and the generation of diacylglyceride, an activator of protein kinase C (PKC), and inositol 1,4,5-trisphosphate. G_o is abundant in neuronal, neuroendocrine, and endocrine cells (45). G_o activity is linked to the activation of ion channels such as Ca^{2+} , Cl^- , or K^+ . G_z was discovered later than other G-proteins (46). It is involved in the inhibition of cAMP production (47), cellular proliferation (48), and the induction of NK cell cytotoxicity (49).

The crystal structure of the heterotrimeric G_i (50) or G_i (51) reveals the complex interaction among the G-protein subunits. The carboxy-terminal helix (and, in some cases, the amino-terminal helix) of the α subunit binds the third transmembrane domain of the heptahelical receptor, but bindings may also occur through the serine/threonine-rich carboxy terminal. The α subunit is anchored in the membrane through its lipid modification: α_s is palmitoylated, α_i is palmitoylated as well as myristoylated, and α_q is myristoylated (52). The γ subunit is either farnesylated or geranylgeranylated, and binds the membranes through its carboxy helix. The β subunit present in

a seven-bladed propeller structure is always associated with the γ subunit (50, 51, 53). It appears that the $\beta\gamma$ complex binds the amino-terminal region of the α subunit, particularly to a site called the switch II region. The same site binds GDP when the receptors are not occupied by the ligands (Fig. 1). However, upon binding of ligands such as chemokines to their putative receptors, conformational changes occur in these heptahelical receptors that allow separation of the third and the six transmembrane loops, creating a pocket and resulting in a higher affinity binding of the α subunit to these receptors. This will induce conformational changes in the α subunit of G-proteins that result in exposure of the switch II region to the membranes, allowing GTP, which is abundant in these membranes, to bind this region. When this occurs, the GTP displaces the $\beta\gamma$ subunit of the heterotrimer from the α subunit (Fig. 1). Both the α (which now associates with GTP) and $\beta\gamma$ subunits are released and activate various effectors that have multiple functions, as shown in Fig. 1. To turn off this activation, the α subunit, which possesses an intrinsic GTPase activity, digests the GTP into GDP, resulting in reassociation of the α subunit with the $\beta\gamma$ complex, hence reverting to the inactive state where the heterotrimer binds the heptahelical receptor waiting for another round of activation.

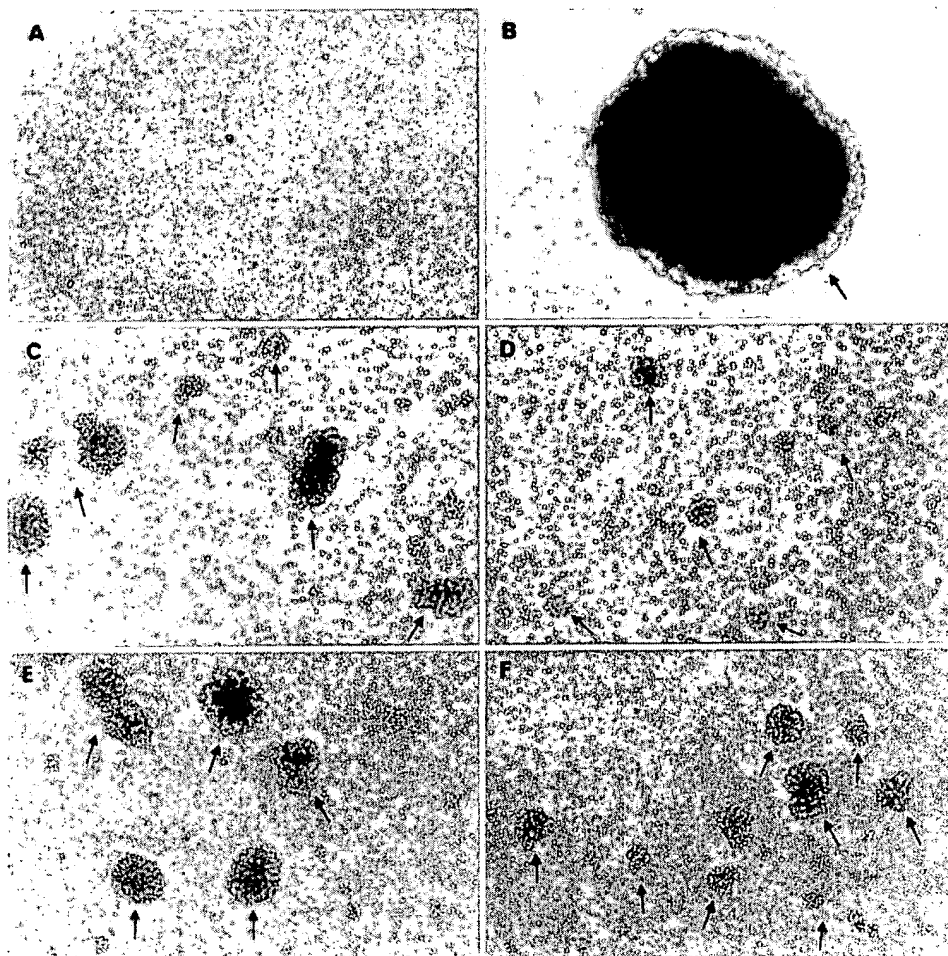


Figure 3. Morphological differences between CHAK and IANK cells. CD56⁺ NK cells were cultured for 10 days with medium only (A), 500 IU/ml IL-2 (B), 10 ng/ml MIP-1 α (C), MIP-1 β (D), MCP-1 (E), or RANTES (F). Notice that CC-chemokine-activated NK cells form clusters that are smaller in size and more numerous than IL-2-activated NK cells.

The presence and identity of the G-proteins in human and rat NK cell membranes have been investigated. It was reported that these membranes expressed G_i, G_o, G_s, G_q, G₁₂, and G₁₃ (49, 56–58). Because it is difficult, if not impossible, with present technology to transfect NK cells with cDNA encoding G-proteins, we developed a method to introduce anti-G-protein antibodies inside permeabilized, IL-2-activated NK cells, using streptolysin O (SLO) as a permeabilizing agent. This procedure facilitates dissecting the functional coupling of G-proteins with chemokine receptors or with intracellular effectors. In this series of experiments, we observed that permeabilization did not alter the function of IL-2-activated NK cells (31, 34, 49, 57). Using this procedure and other methodologies, we showed that G-proteins play vital roles as early transducers of various biological functions in NK cells:

1) the chemokinetic activity of IL-8 for IANK cells is linked to the activation of G-proteins, particularly G_o (12); 2) the chemotactic activity of transforming growth factor β 1 for rat IL-2-activated NK cells is linked to the activation of G-proteins, particularly G_s and G_o (56); 3) the chemotactic activity of CC chemokines for human IANK cells is due to the activating

of all subtypes of G-proteins (59); 4) the chemotactic effects of IP-10 and lymphotactin for human NK cells is linked to the activation of G_o, G_i, and G_q (31); and 5) the chemotactic effect of SDF-1 α for human NK cells requires the activation of G_o and G_q (34).

We suggested that the $\beta\gamma$ subunit of G-proteins may be involved in chemokine-induced NK cell chemotaxis (31). Recent work with transfected cell lines supported this view and showed that the $\beta\gamma$ subunits of G-proteins play important roles in transducing the chemotactic signals induced by chemokines and non-chemokine peptides (60, 61).

Receptors for the CC chemokines MIP-1 α , MCP-1, and RANTES (59), the C chemokine lymphotactin, and the CXC chemokines IP-10 (31) or SDF-1 α (34) are promiscuously coupled to several heterotrimeric G-proteins in NK cells. Kuang et al. (62) observed similar promiscuous coupling of CC chemokine receptors to G-proteins in cotransfected COS-7 cells. Also, Tanabe et al. (63) reported that SDF-1 α receptors are coupled to multiple G-proteins in astrocytes and microglial cells. Perhaps this promiscuous coupling is developed by the cells to ensure maximum and guaranteed transmission of the signals generated when the ligands bind their receptors. A cross-talk

between multiple G-proteins at the postreceptor level has previously been suggested (64–67). For example, the α subunit of G_z (66) or G_{12} (67) is phosphorylated and inhibited by PKC generated upon the activation of phospholipase C β by G_q . Recently it was shown that the signal induced by β_2 -adrenergic receptors can switch between G_i and G_s , depending on the activation of second messengers such as protein kinase A (68). These results confirm the cross-talk principle among the various subunits and subtypes of the heterotrimeric G proteins. In NK cells, PT (which uncouples G_i or G_o from the membranes) inhibits the chemotactic response induced by several chemokines (31). However, this does not mean that G_q (or other PT-insensitive G-proteins) is not involved in chemokine-induced chemotaxis, since there may be cross-talk between the α subunit of G_q or its $\beta\gamma$ complex with the α subunits of G_i and/or G_o or their $\beta\gamma$ complexes (31). The reciprocal dependence of G_i or G_o on G_q is also true. To summarize, there is a strong indication that chemokine receptors are coupled to multiple heterotrimeric G-proteins in NK cell membranes. In addition, a cross-talk among the various subunits and subtypes of these G-proteins is obligatory for initiating the motility of NK cells.

OTHER INTRACELLULAR SIGNALING PATHWAYS

In addition to G-protein-coupled receptors (GPCR), receptor tyrosine kinases (RTK) transmit intracellular signals resulting in the activation of common second messengers, such as: 1) intracellular calcium, 2) mitogen-activated protein kinase (MAP kinase, also known as extracellular-regulated kinase, or ERK), and 3) phosphoinositide-3 kinase (PI-3K), among others. **Figure 2** shows the various components of these pathways. Tyrosine kinases that are associated with but not coupled to the receptors are known as nonreceptor tyrosine kinases. About 10 subfamilies of these kinases have been described: Src, Brk, Abl, Fes, Syk, Tec, Jak, Fak, Ack, and Csk. The most extensively studied in T lymphocytes include members of the Src and Syk subfamilies (69). c-Src, c-Yen, Fyn, Yrk, c-Fgr, Lyn, Lck, Hck, and Blk belong to the Src subfamily, whereas Syk, Zap-70, and HTK-16 belong to the Syk subfamily. Phosphorylation of Syk and Zap-70 has been reported after perturbation of the Fc (70) or CD94 receptors (71) in NK cells. Recently, the adaptor molecule DAP12 involved in NK cell activation was found to be associated with Syk and ZAP-70 (72). The CC chemokine RANTES induces phosphorylation and the association of ZAP-70, paxillin, and focal adhesion kinase p125^{FAK} in T cells (73). Whether RANTES induces the phosphorylation of these molecules in NK cells has not been examined, but will be an important issue because of the ability

of NK cells to adhere to the microvascular endothelium. Nor is it known whether chemokines activate members of the Src and Syk subfamilies. These are important areas for future research.

MAP kinases (ERK1 and ERK2) phosphorylate various nuclear binding proteins such as c-Jun/Fos, c-Myc, and ELK-1, among others, resulting in gene expression and mRNA and protein synthesis. This pathway is therefore implicated in the proliferation of various cell types and may be involved in the proliferation of NK cells and their cytolytic activity. MAP kinase is phosphorylated by another kinase, known as MAP kinase kinase (ERKK or MEK), which in turn is phosphorylated by a serine/threonine kinase known as Raf-1 (MAP kinase kinase kinase). Raf-1 was found to be a substrate for the small GTP binding protein p21^{ras}. The latter is activated by a homologue of Sos-1, which is recruited to the cell membrane by an adaptor protein called Grb-2, a 23 kDa protein having three Src homology domains (SH). Through its SH3 domains it binds the carboxy terminal of Sos, whereas through its SH2 it binds the adaptor molecule Shc, which exists in three different forms, 46, 52, and 66 kDa. Dimerization of various receptors activates Shc, resulting in the activation of the p21^{ras} pathway. Also, the $\beta\gamma$ subunit of the heterotrimeric G_i activates Shc, with subsequent activation of Grb2, Sos-1, p21^{ras}, and MAP kinase (74). The α subunit of G_q (75) and G_o itself (76) are also involved in activating the MAP kinase pathway. Galandrini et al. (77) showed that activation of human NK cells by ligating the CD16 molecules results in the phosphorylation of Shc and its association with the SH2 domain of Grb-2, which leads to the eventual activation of p21^{ras}. However, it is not known whether stimulation of NK cells with chemokines will result in a similar stimulation of the p21^{ras}/MAP kinase pathway.

PI-3K is present in three different forms: I, II, and III. These enzymes phosphorylate the D3 position of phosphatidylinositol to produce phosphatidylinositol 3-phosphate, phosphatidylinositol 3,4-bisphosphate, or phosphatidylinositol 3,4,5-trisphosphate (78). PI-3K I is present in mammals, and is divided into PI-3K IA and PI-3K IB (79). IA is composed of a catalytic subunit (P110 α , β , or δ) that is associated with a regulatory p85 subunit. The latter possesses an SH2 domain that binds the phosphorylated receptor upon activation. On the other hand, IB expresses the catalytic p110 γ in association with the adaptor molecule p101 (80). The p110 γ subunit and its regulatory protein lack the SH2 domain; hence, they do not bind the tyrosine-phosphorylated receptors, but instead have a motif that binds the $\beta\gamma$ subunit of G-proteins (81). This subunit plays a major role in the activation of MAP kinase pathway (82). Recent studies have shown that PI-3K γ activates the Jun kinase through the $\beta\gamma$ subunit of G-proteins (83). The involvement

TABLE 2. Chemokine receptors^a

	Binds to	Coreceptor or receptor for
CXCR		
CXCR1	IL-8	
CXCR2	IL-8, GRO- α , - β , - γ , NAP-2, ENA, GCP-2	
CXCR3	MIG, IP-10	
CXCR4	SDF-1	T-tropic HIV-1
CXCR5	BCA-1	
CCR		
CCR1	MIP-1 α , RANTES, MCP-2, -3	
CCR2a	MCP-2, -3, -4	
CCR2b	MCP-1, -2, -3	M-tropic HIV-1
CCR3	Eotaxin, RANTES, MCP-3, -4	M-tropic HIV-1
CCR4	TARC, MDC	
CCR5	MIP-1 β , MIP-1 α , RANTES	M-tropic HIV-1
CCR6	MIP-3 α	
CCR7	MIP-3 β	EBV, BL
CCR8	I-309	
CX₃CR		
CX ₃ CR1	Fractalkine	
Orphan receptors		
STRL33/Bonzo		M-tropic HIV-1, T-tropic HIV-1, SIV-1
BOB/GRP15		M-tropic HIV-1, T-tropic HIV-1, SIV-1
Shared receptor		
Duffy antigen	CC and CXC chemokines	<i>Plasmodium vivax</i>

^a HIV-1, human immunodeficiency virus 1; EBV, Epstein Barr virus; BL, Burkitt's lymphoma; SIV-1, simian immunodeficiency virus 1.

of PI-3K in the chemotaxis of T cells was suggested by Turner et al. (84), who observed that wortmannin, a fungal inhibitor of PI-3K, inhibits RANTES-induced T cell chemotaxis. Because wortmannin inhibits all forms of PI-3K, it is not clear which isotype is involved in this chemokine activity. Recently we observed that wortmannin and inhibitory antibodies to PI-3K γ inhibit C, CC, and CXC chemokine-induced NK cell chemotaxis (unpublished observations), suggesting that PI-3K IB plays an important role in chemokine activation of NK cells.

CHEMOKINES ACTIVATE NK CELLS AND INHIBIT THE REPLICATION OF HIV-1

Chemokines not only induce the chemotaxis of NK cells, but also induce the mobilization of intracellular calcium in these cells (29–31, 34). It appears that chemotaxis is always associated with chemokine-induced calcium fluxes in NK cells; treatment of NK cells with PT or with inhibitory antibodies to G-proteins incorporated into SLO-permeabilized NK cells inhibits both activities to the same extent (31, 34). In addition, CC chemokines induce granule exocytosis in NK cells (29, 30). This is an important finding, since proteolytic and other lytic enzymes secreted by NK cells upon binding their target cells play major roles in NK cell-mediated cytotoxicity. Several groups observed that CC chemokines activate NK cells to kill tumor target cells (29, 85). We observed that the CC

chemokines MIP-1 α , MCP-1, RANTES, and to a lesser extent, MIP-1 β , enhance the cytotoxicity of NK cells when cultured with these cells for 10–14 days (86). These cells are designated as CHAK (CC chemokine-activated killer) cells to distinguish them from IANK cells, since they show distinct morphological differences. Whereas IANK cells strongly adhere to each other, CHAK cells adhere to a much lesser extent, forming clusters that are more numerous in cultures (Fig. 3).

At almost the same time it was observed that the CC chemokines MIP-1 α , RANTES, and MIP-1 β inhibit the replication of non-syncytium-forming (M-tropic) HIV-1 (87). Also, the CXC chemokine SDF-1 α inhibits the replication of syncytium-forming (T-tropic) HIV-1 (88, 89). It was suggested that the anti-HIV-1 activity of chemokines is a passive mechanism due to binding of chemokines to their receptors, which also act as HIV-1 coreceptors (see Table 2). Hence, the ability of MIP-1 α , MIP-1 β , or MCP-1 to inhibit the replication of M-tropic HIV-1 strain is due to the ability of these chemokines to bind CCR5 (90–92), CCR2b (93), or CCR3 (93, 94), which act as this strain's coreceptor present on CD4⁺ cells. In addition, the coreceptor for the T-tropic HIV-1 strain was identified and designated as fusin (95); later it was designated CXCR4 (88, 89). This receptor binds the CXC chemokines, SDF-1. An association among chemokines, chemokine receptors, and suppression of HIV-1 replication has been established, but the mechanisms of this suppression have not.

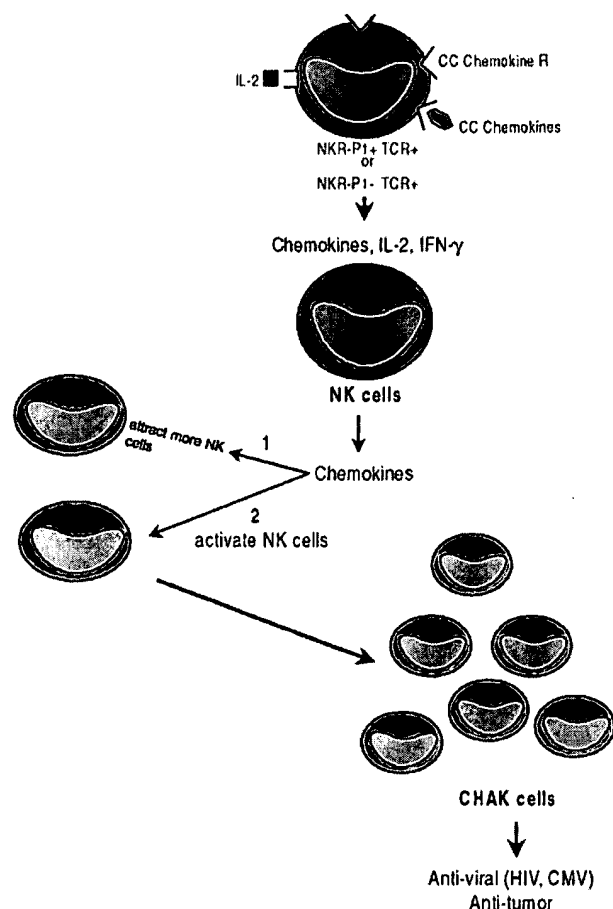


Figure 4. Proposed mechanism of recruitment of NK cells toward the sites of tumor metastases or viral infection. T cells (TCR^+) or NK-T cells (TCR^+ , NKR-P1^+ , and human equivalent) express chemokine receptors after exposure to IL-2. These cells secrete various cytokines and chemokines that induce chemotaxis (chemokines) and activation (chemokines, IL-2, $\text{IFN-}\gamma$) of these cells. NK cells also secrete various cytokines, including chemokines, which perform two functions: 1) the recruitment of more NK cells, and 2) their activation. Activated NK cells will induce the destruction of tumor cells and virally infected cells, probably through direct contact rather than through an indirect mechanism, such as the secretion of $\text{IFN-}\gamma$. CMV, cytomegalovirus.

Paxton et al. (96) reported that some individuals exposed to HIV-1 are not infected with the virus. To explain this finding, it was suggested that some of these individuals have a mutation in the CCR5 allele (97). In addition, Smith et al. (98) observed that a mutation in the CCR2 or a combination of CCR2 and CCR5 mutations results in delayed progression toward AIDS, although these mutations do not affect the incidence of HIV-1 infection. These findings led to speculation that when chemokine receptors are not expressed on the cell surface, HIV virus will not infect these cells, and suggested that the anti-HIV-1 activity of chemokines is related to the ability of these molecules to bind the chemokine receptors, hence competing out the HIV-1 strains. This raised the idea

that blocking chemokine receptors will lead to the treatment of HIV-infected individuals (99). Several groups (100–102) developed small molecules that bind virus coreceptors, whereas others targeted these receptors in the endoplasmic reticulum, inhibiting their expression on the cell surfaces (103, 104). It was suggested that these strategies be used to treat AIDS patients. However, this hypothesis has been challenged by other investigators, who described HIV-1 patients who are homozygous for CCR5 mutation, yet are infected with the virus (105, 106). Similarly, a mutation in the CCR2 gene is not associated with reduced HIV infection or slower progression toward AIDS (107). These results suggest that CCR5 or CCR2 down-regulation may not be the only important factor in CC chemokine inhibition of HIV-1 replication. Supporting this concept is the recent finding by Pal et al. (108) showing that MDC, a recently described CC chemokine, inhibits the replication of M-tropic as well as T-tropic HIV-1 strains. MDC does not bind the HIV-1 coreceptors CCR5, CCR2b, CCR3, or CXCR4, but it binds CCR4 (109), which is not a coreceptor for this virus. Most important, MDC activates NK cells (35; see Table 1).

It can be concluded that receptor blockade does not completely explain the anti-HIV activity of chemokines. What, then, are the other factors contributing to this activity? Cocchi et al. (87) suggested that the inhibitory effect of chemokines may be either direct or indirect through the recruitment of effector cells in the proximity of the infection foci. In addition, Furci et al. (110) observed that in HIV-1-exposed noninfected individuals, T lymphocytes secrete high levels of CC chemokines. Therefore, it can be postulated that in these individuals, T helper and perhaps other cells such as NK-T cells, which are known to respond to and secrete various chemokines (111, 112), chemoattract and activate the anti-viral effectors NK cells. These cells inhibit replication of the virus by direct contact with virally infected cells 'active mechanism' (Fig. 4). The ability of NK cells to kill gp120-transfected cells has previously been shown (113). Also, NK cells kill the Rauscher murine leukemia virus-infected cells in the murine AIDS model (114).

One may argue that most experiments describing the anti-HIV activity of chemokines have been performed *in vitro*, where presumably there are no NK cells (although this is not known for certain). Nonetheless, evidence showing the activation of NK cells by chemokines and the strong anti-viral activity of these cells support the notion that the mechanism described here may be taking place *in vivo*. This also suggests that blocking chemokine receptors may have adverse effects, since such treatment would impede activation of the anti-HIV effector cells expressing receptors for chemokines. AIDS patients suffer from multiple opportunistic infectious diseases.

Therefore, the presence of the activated effector cells may contribute to combating these infections. Any therapeutic modality, whether intended to target the chemokine receptors or not, should take into consideration the vital aspect of activating the potential anti-HIV effector cells.

CONCLUDING REMARKS

The ability of chemokines to activate the anti-tumor/anti-viral effector NK cells may be important for the treatment of cancer and AIDS patients. The finding that CHAK cells form smaller clusters than IANK (LAK) cells suggests that CHAK cells are suited for the immunotherapy of cancer patients. CHAK cells may have better migratory behavior than LAK cells, since they may extravasate into various tissues and may not clog the vessels as do LAK (IANK) cells. In addition, CHAK cells may not need the continuous infusion of the stimulatory chemokine. This is a drawback of therapy with LAK cells since IL-2, which proves to be toxic if administered in high doses, is needed to support LAK cells in both clinical and pre-clinical trials. In addition, the ability of chemokines to attract and activate NK cells at the sites of viral infection, combined with their ability to inhibit the replication of HIV-1 in infected cells, suggests that NK cells play significant roles in the eradication of virally infected cells. The ability of chemokines to activate various intracellular signaling pathways in NK cells provides an understanding of the migratory behavior of these cells and their activation, and should facilitate the planning of strategies for using NK cells for therapeutic purposes.

The field of signal transduction in NK cells is in its infancy. Future research should examine the coupling of chemokine receptors to multiple heterotrimeric G-proteins and the roles that the $\beta\gamma$ subunits of G-proteins play in various biological functions exerted by NK cells. Investigations of whether chemokines activate the PI-3 kinase pathway, the MAP kinase pathway, or the nonreceptor tyrosine kinases are highly important, and may lead to therapeutic and preventive interventions. We have focused on the ability of chemokines to activate NK cells. Information regarding the effects of chemokines on other cell types and the roles of chemokines in inflammation can be found in other recent review articles (25, 115, 116).



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